NOVEL GENE ENCODING ORGANIC ANION TRANSPORTER

Background of the Invention

This invention relates to the processing of anions by the human kidney. In particular, it relates to nucleic acids and polypeptides transcribed and expressed in the kidneys that are responsible for the removal of toxic anions from the circulation.

Sweet et al. have described a rat gene (ROAT1) encoding an organic anion transport protein expressed in the rat kidney (*Journal of Biological Chemistry* 272:30088-30095 [1997]). Sekine et al. also appear to have disclosed essentially the same gene, denominated OAT1 (*Journal of Biological Chemistry* 272:18526-18529 [1997]). Its apparent murine counterpart has been described in Lopez-Nieto et al. *Journal of Biological Chem.* 272:6471-6478 (1997).

It is an object of this invention to isolate nucleic acid encoding a human organic anion transporter (hOAT).

It is another object of this invention to recombinantly express nucleic acid encoding hOAT.

Another object is to obtain hOAT expressed in elevated yields in recombinant cell culture.

An object is to prepare isolated polypeptide encoding hOAT.

An additional object of this invention is to provide novel hOAT

polypeptides.

A further object of this invention is an assay for evaluation of potential drug-drug interactions due to interference of one drug or group of drugs with hOAT-mediated active kidney excretion of another drug.

5   A further object of this invention is an assay system for screening candidate compounds that agonize or antagonize the expression of hOAT and/or the biological activity of hOAT polypeptide, especially its anion transport activity.

Another object is to provide an hOAT screening assay system to identify

10   molecular variants of nephrotoxic compounds that will be taken up and transported by hOAT to a lesser degree than is the case with the parental compound.

A further object is to identify alleles or isoforms of hOAT that are associated with sensitivity to nephrotoxic compounds, particularly nephrotoxic

15   drugs.

These and other objects of the invention will be apparent from consideration of this specification as a whole.

SUMMARY OF THE INVENTION

20   In accordance with the objects, applicants provide isolated nucleic acid encoding hOAT and isolated hOAT polypeptide. hOAT is expressed in recombinant cells, where it finds use in evaluating compounds for nephrotoxicity or for identifying compounds having the ability to prevent nephrotoxicity. Since hOAT is expressed in human brain, it also finds use in

25   characterizing compounds known or suspected to influence brain function. hOAT also is useful in a method for identifying any hOAT alleles and isoforms which are correlated with patient sensitivity to nephrotoxic agents.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 (SEQ. ID NOS. 1 and 2) depicts a nucleotide sequence for cDNA encoding hOAT and its translated sequence.

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DETAILED DESCRIPTION OF THE INVENTION

hOAT polypeptide is defined as a polypeptide sequence that is at least about 85% homologous by amino acid sequence (ordinarily at least about 90%,

10 and prefereably at least about 95%) with the sequence of Fig. 1 (reference sequence hOAT).

An expressed sequence tag (EST) of approximately 200bp having high homology to a segment of hOAT is found in the GenBank EST entries under accession no. R25797. Invention hOAT nucleic acids *per se* as defined herein

15 exclude any expressed sequence tag (EST) or other nucleic acid sequences found in public databases on the filing date (such databases being expressly incorporated by reference), including the sequence of accession no. R25797 as well as the rat ROAT1 and OAT1, and mouse NKT, sequences of the prior art. However, other inventive subject matter such as isolated protein, methods for

20 screening and the like as set forth above do not (unless expressly stated to the contrary) exclude the use of the R25797 sequence or its expression product.

"Homology" is defined as the percentage of residues in a candidate amino acid sequence that is identical with the residues in the reference sequence hOAT after aligning the two sequences and introducing gaps, if necessary, to

25 achieve the maximum percent homology. Methods and computer programs for the alignment are well know in the art. One computer program which may be used or adapted for purposes of determining whether a candidate sequence falls within this definition is "Align 2", authored by Genentech, Inc., which was

filed with user documentation in the United States Copyright Office, Washington, DC 20559, on December 10, 1991.

"Isolated" hOAT nucleic acid is one that has been separated from its environment as it is found in nature, i.e., from the genome in the case of DNA

5   or from cellular environment in the instance of RNA.

"Isolated" hOAT polypeptide is one that has been separated from its normal cellular environment, and includes hOAT protein that is homogeneous by SDS-PAGE using silver stain.

In calculating amino acid sequence homology the candidate and

10   reference sequences are aligned in the fashion that produces the maximum number of aligned residues, with insertions and deletions of residues represented by gaps in the aligned sequences. For example, a 120 residue polypeptide containing a 100 residue reference sequence fragment fused at its N-terminus to a 6 residue polyhistidine affinity tag, but with a single

15   substitution in the hOAT domain, is calculated to be 99% homologous to the hOAT reference sequence since the sequence of the fragment corresponds exactly to the maximally aligned hOAT reference sequence except for a single residue substitution and the 6 residue N-terminal fusion. Thus, if the alignment-maximizing comparison of the candidate and reference sequences

20   reveals an insertion (or deletion) of one or more amino acid residues, then these residues are ignored for the purposes of making the homology calculation. Applicant recognizes that this convention gives rise to theoretical 100% homology between 2 differing sequences, but has chosen to establish his own definition for the purposes of this filing.

25   Analysis of homology is based on any one or more of the sequences imputed from the nucleic acid used to express the hOAT, the sequence of the product as first produced *in vitro*, or the sequence after any post-translational modification. Thus, if the reference and candidate sequences are identical when

expressed, but a glutamine residue is later deaminated to glutamic acid, the first candidate is 100% homologous, but the deaminated sequence is not.

For the purposes herein "hOAT activity" means any one or more of the functions performed by hOAT in the human, including in particular the

5   transport of organic anions.

It is not necessary for a polypeptide to have anion transport activity in order to fall under the definition of hOAT herein. For example, in some embodiments hOAT polypeptides possess at least one immune epitope that is capable of substantial cross-reaction with an antibody raised against reference

10   sequence hOAT, and thus are useful in immunoassays for hOAT, but may possess mutations that render the polypeptide incapable of anion transport.

The hOAT polypeptides of this invention comprise substitutions for, deletions of, or insertions of any amino acid residue adjacent to any of the reference sequence amino acid residue sites shown in Fig. 1. Substitutional

15   hOATs are those in which at least one amino acid residue in the reference sequence has been removed and a different amino acid inserted in its place at the same position. One or more residues are substituted.

Alanine is a common substitution for any residue, and is commonly used in alanine scanning to identify functional residues, but it is within the scope of

20   this invention to substitute other residues into the hOAT reference sequence. The introduced residues generally are naturally occurring amino acids, commonly G, A, Y, V, L, I, S, T, D, E, Q, C, M, N, F, P, W, K, R or H (using conventional single letter code; EP 323,149). Suitable residues also include hydroxyproline, beta-hydroxyaspartic acid, gamma-carboxyglutamic acid,

25   hydroxylysine or norleucine, to be employed as alternatives to their namesakes.

These substitutions may be conservative, i.e., the substituting residue is structurally or functionally similar to the substituted residue. Other substitutions will be less conservative in that they constitute an exchange between different structural or functional classes of residues. For the purposes

herein, these classes are as follows: 1. Electropositive: R, K, H; 2. Electronegative: D, E; 3. Aliphatic: V, L, I, M; 4. Aromatic: F, Y, W; 5. Small: A, S, T, G, P, C; 6. Charged: R, K, D, E, H; 7. Polar: S, T, Q, N, Y, H, W; and 8. Small Hydrophilic: C, S, T. Intergroup substitutions generally will have greater

5   effects on protein function than conservative (intraclass) substitutions. Thus, it is particularly within the scope of this invention to introduce conservative substitutions into hOAT and, if the results are not satisfactory, to introduce non-conservative substitutions at the sites. Typically, however, proline, glycine, and cysteine substitutions or insertions into the sequence are not favored. An

10   example of an expressed variant is a change at codon 498 from AGC to ATC, resulting in expression of isoleucine in place of serine. Other variants are introduced into DNA encoding hOAT without resulting in a change in protein sequence, e.g. from ATC to ATT at codon 453 or from GGG to GGT at codon 491.

15   hOAT variants are readily identified by methods apparent to the ordinary artisan. For example, sites shown by alanine scanning to influence selected biological activity are subjected to saturation mutagenesis to identify the optimal modification for the activity in question, e.g. selectivity for transport of a particular anion.

20   hOATs representing combinations of sequence variants are within the scope of this invention. 2, 3, 4, 5, or more substitutions, deletions or insertions are introduced into hOAT as defined herein. Typically, a deletion of a single residue will be accompanied by an insertion within 1 to about 3 residues of the deletion site. Generally, deletions of larger domains unnecessary for anion

25   transport activity need not be accompanied by an insertion. The results of individual amino acid substitutions are generally additive except when the residues interact with each other directly or indirectly. They are readily screened using the same procedures described in Sweet et al. or Sekine et al.

(*supra*) in order to identify those having the properties of reference sequence hOAT or the desired modified properties.

Included within the scope of this invention are hOATs having one or more amino acids inserted immediately adjacent to a hOAT amino acid at any position in the reference sequence. Insertional hOATs generally will have a polypeptide structure comprising the sequence NH_2-PP-A-$(X)_{n1}$-B-PP-COOH, wherein X is the inserted residue(s) (which may be the same or different), n1 is an integer (generally 1-30, typically 1 or 2), either A or B are the designated residue sites for insertion and PP represents the remainder of the hOAT or a bond at the hOAT N or C terminus.

The invention includes fusions of hOAT and selected antibody recognition sequences (heterologous polypeptides) for immunoaffinity purification of hOAT from cell culture, fusions of hOAT sequences with affinity tags such as FLAG or polyhistidine, and chimeric sequences (particularly fusions of hOAT sequence fragments with fragments of other receptors of the 12-transmembrane spanning region class).

Also included within the scope of this invention are hOATs in which a glycosylation site is introduced or removed from the reference sequence, whether by substitution, insertion or deletion of one or more amino acid residues. Such changes will result in the installation or removal of the sequence NXS or NXT, where X can be any residue. Thus, asparagine can be substituted for any residue located 2 residues N-terminal to serine or threonine to introduce a glycosylation site. Alternatively, single glycosylation can be omitted by substituting glycosylated asp with any residue, deleting site-adjacent serine or threonine substituting any residue into the glycosylation site to perturb the NXS or NST sequence.

Also included within the scope of this invention are deletional hOATs, i.e., hOATs in which one or more amino acid residues of the reference sequence

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have been removed at a designated site, whereby flanking residues are now joined by a peptide bond in the ordinary fashion. It generally is not preferred to delete P, C or G residues.

Typically, deletions or insertions are relatively small, on the order of 1 to 10 residues and generally no more than 2, although deletions or insertions can be quite large if they are not in critical portions of the reference sequence, or the additional sequence is to be removed at some point during post-translational or post-recovery processing. The number of residues that are deleted or inserted in part will depend upon whether or not they are found in secondary structural components such as helices or sheets (whereupon only 1 or, preferably 2 residues are inserted or deleted), or are in less structurally confined domains such as loops, where larger numbers of residues may be deleted or inserted without unduly perturbing the structure of hOAT.

The hOATs of this invention may be subject to post-translational covalent modification, e.g. deamidation of asparagine or glutamine, or oxidation of cysteine residues. Glycosylation can be variant or absent depending upon the host cell used to express the variant. hOATs containing such modifications are included within the scope of this invention. If hOAT is glycosylated in recombinant cell culture, it preferably is glycosylated with carbohydrates characteristic of mammalian cells, although it also may bear fungal (such as yeast) glycosylation patterns. Glycosylation is acceptable which is characteristic of expression of hOAT from one or more of fibroblast, kidney, brain, lung, skin, neural, liver or bone marrow cells or cell lines, or from any mammalian cell line such as CHO or embryonic kidney cells.

Naturally occurring human alleles are included within the scope of this invention. These readily are identified by obtaining nucleic acid samples from individuals in a population, sequencing hOAT from such individuals and determining residues at which variation is found. Once each variation is identified, it is straight-forward to determine the frequency of the putative

allele in other individuals by PCR using primers specific for the domain in question, or such other methods as are conventional in the field for determining proportions of alleles in human populations.

5   Preparation of hOAT Polypeptides

The hOATs of this invention are readily prepared by methods known in the art. In general, nucleic acid encoding the hOAT is prepared by PCR, by *in vitro* synthesis (Vasser et al., "N.A.R." 18(a0):3089 [1990]), by cloning from a
10   human genomic or kidney cDNA library or combinations thereof. Site-directed mutagenesis of hOAT-encoding nucleic acid is used to prepare nucleic acid encoding sequence variants. The + (coding) and – strands (antisense) of hOAT are included within the scope of hOAT nucleic acids, as are hOAT-encoding cDNA, genomic DNA and RNA. hOAT DNA includes 5' and 3' regions of the
15   hOAT gene that are not transcribed but serve as transcription control domains, and transcribed but not expressed domains such as introns (including splice junctions), polyadenylation signals, ribosomal recognition domains and the like.

The hOAT nucleic acid is expressed in *in vitro* systems or in recombinant host cells. One method for expression is ribosome based synthesis using
20   dedicated tRNAs (Benner, "TIBTECH" 12:158-163 [1994] and Robertson et al., "J. Am. Chem. Soc." 113:2722-2729 [1991]). Ordinarily, however, the hOAT-encoding nucleic acid (generally DNA) is inserted into an appropriate expression vector recognized (transcribed and translated) by the host cells, host cells are transfected with the expression vector, the recombinant host cells are
25   grown in suitable culture medium, and optionally the desired hOAT is recovered from the recombinant cell culture by chromatographic or other purification methods. It is also within the scope of this invention to partially synthesize the hOAT in recombinant cell culture or by *in vitro* methods and then

ligate the polypeptide fragments by peptide ligase (reverse proteolytic synthesis).

The nucleic acid for expression of the hOAT may comprise an exogenous signal sequence. Here, the hOAT is expressed as a preprotein of hOAT,

5   whereby the hOAT is expressed as a precursor that is processed to mature hOAT. Suitable presequences include those of (a) microbial proteases such as subtilisin, (b) mammalian polypeptides, (c) cytokines such as gamma interferon or an interleukin, (d) growth factors such as growth hormone or TGF-alpha, (e) polypeptides or proteins having N-terminal mature sequences that are

10   homologous to human hOAT, (f) immunoglobulins, (g) receptors, or (h) other presequences of secreted or cell membrane bound proteins. Signal sequences optionally are derived from or are homologous to the host cell, or at least the phylogenetic branch to which the host cell belongs. For example, one ordinarily would use a presequence of a yeast protein, such as mating factor, in a yeast

15   expression system, or of a bacterium, such as ST-II or beta-lactamase, in bacterial cell culture systems. A wide variety of suitable signal sequences are known and can be used in methods for the preparation of the hOATs described herein.

The nucleic acid constructs encoding hOAT generally are spliced into

20   expression vectors where they will be under the control of sequences controlling transcription, translation and RNA stability. These sequences include promoters, operators, enhancers and polyadenylation sequences, and are generally known in the art. Constructing suitable expression vectors for hOATs of this invention is a matter of routine for those skilled in the art, and

25   will be accomplished using the conventional tools of molecular biology, including nucleic acid synthesis *in vitro*, PCR, adapters, ligases, restriction enzymes, expression and shuttle plasmids, transfection aids and the like, all of which are publicly (and for the most part commercially) available.

Suitable host cells for transfection with nucleic acid encoding hOAT are well known. Some are mentioned above while others are described in WO 93/13208 at page 12, line 21 – page 19, line 5, and EP 319,312 B1, page 16, lines 10-18 and Table II thereof. It may be optimal to use host cells that are capable of

5   glycosylating hOAT, typically including mammalian cells such as embryonic kidney 293 cells, COS cells, CHO, BHK-21 cells and the like. *Xenopus* oocytes are suitable for expression of hOAT RNA. In addition, host cells that have been used heretofore to express anion transporter polypeptides in recombinant cell culture are suitable.

10   The host-vector system should yield substantially homogeneous hOAT, thereby avoiding the need to purify various hOAT alleles, isoforms or cleavage products from one another. If the host cell is capable of glycosylation, essentially all of the hOAT molecules should be glycosylated. In addition, host cells optimally will not proteolyse hOAT. Cells can be selected that contain no

15   protease, e.g., in the periplasm, that will cleave hOAT. For example, *E. coli* and other microbial strains are known that possess little or no extracellular or periplasmic proteolytic activity (other than signal peptidases). The absence of deleterious proteases helps to ensure that the product is not rendered microheterogenous as to chain length by host-endogenous proteases acting on

20   the hOAT expression product. In addition, or alternatively, basic residues of hOAT that define sites for proteolytic cleavage are substituted with residues other than K or R.

The hOAT recombinant cells are cultured under conventional conditions using conventional culture media heretofore employed with the cells in

25   question. These conditions and media are widely known. Freshly transfected cells may only transiently express the hOATs, but stable transformants readily are obtained in accord with conventional practice using cotransformation with a selection gene such as DHFR or glutamine synthetase and serial culture in the presence of a selection agent such as methotrexate or methionine sulfoximine,

respectively. Yields of hOATs can differ substantially despite minor differences in the character of substituents or insertions. In such cases, it is desirable to screen for an expression system that will yield a quantity of hOAT that is at least about 75% of that obtained with the reference hOAT in the same

5   expression system.

The hOAT may be expressed in bacteria in the form of retractile bodies, in which base the insoluble hOAT is recovered and refolded using known methods, e.g. dissolution in a denaturant such as guanidinium hydrochloride followed by gradual removal of the denaturant. Directly expressed hOATs of

10   this invention may have an extra N-terminal methionine or blocked methionine residue, although host cells can be employed that will cleave away such N-terminal methionine residues if they are extraneous to the protein as found in nature. In order to avoid difficulties with insoluble expression products it is preferable to express hOAT in eukaryotic, more preferably mammalian, cells.

15   hOAT is isolated or purified from recombinant cell culture by methods heretofore employed for other proteins, e.g., native or reducing/SDS gel electrophoresis, isoelectric focusing, immobilized pH gradient electrophoresis, salt precipitation, solvent fractionation (using ethanol for example) and chromatography such as gel filtration, ion exchange (cation or anion), ligand

20   affinity (cibacron blue F3GA or p-aminobenzamidine), immunoaffinity, chromatofocusing, reverse phase or hydrophobic interaction chromatography. Typically, the hOAT will be isolated so as to be >95% pure by weight of protein, and preferably greater than 99% pure. However, the term "isolated" as used in reference to hOAT protein or nucleic acid refers to the absence of one or more of

25   the normal human polypeptides or nucleic acids found in association with hOAT in its natural environment, and does not necessarily imply that the hOAT is purified to any degree free of non-hOAT proteins. Since hOAT is normally found in the cell membrane, it is preferable to recover recombinant hOAT as a membrane preparation; otherwise the function of hOAT may be perturbed. In

any case, many utilities for hOAT do not require purification of the protein at all; screening assays for agonists or antagonists are best conducted in intact host cells.

The hOAT polypeptides or their fragments optionally are prepared *in vitro*, especially if they are relatively small, e.g. on the order of about 30 residues or less. For example, hOATs are prepared by synthesis using standard solid-phase peptide synthesis procedures as described by Merrifield "J. Am. Chem. Soc." 85:2149 (1963). These then are ligated together by the use of peptide ligase (reverse proteolysis). *In vitro* methods of protein synthesis also are used to prepare hOATs without the need for recombinant cell culture. Such methods are useful for small-scale preparations, and have the advantage of reducing the possible effect on yields of host cell proteases. *In vitro* hOAT protein synthesis has one additional quite substantial advantage in that it permits the site-specific introduction into the hOAT of a non-naturally occurring amino acid residue. Accordingly, when the term "amino acid residue" is used herein (in connection with hOAT modification by substitution or insertion, especially by a single amino acid) it will be understood that the amino acid is not limited to the naturally occurring residues associated with native tRNAs. Aminoacyl tRNA is efficiently prepared using a variety of non-naturally occurring amino acid residues ("non-naturally occurring" means not naturally found in proteins, although the amino acid might be found elsewhere in nature). Since the tRNA is selected to be incorporated at a codon not recognized by any of the tRNAs normally involved in protein synthesis, the selected non-naturally occurring amino acid residue is incorporated only at the particular site in the hOAT sequence chosen for the unique codon. Thus, in these cases the hOAT is encoded by a nucleic acid having a nonsense codon, e.g., UAG, at the desired unique insertion or substitution site.

Suitable non-naturally occurring amino acids are described for example in Greenstein et al., "Chemistry of the Amino Acids" Vols. 1-3, 1986. In general,

one will use pharmaceutically innocuous L-amino acids that are found in nature but ordinarily not incorporated into proteins. Such amino acids typically will be structurally related to a naturally occurring residue that produces the desired effect at a given site and will be used to further resolve and optimize

5   the desired property of the hOAT.

The hOATs of this invention also include hOATs that have been substituted by a non-peptidyl moiety, either for purposes of preparing the hOAT to begin with or as a subsequent modification of the hOAT prepared by amino acid substitution, insertion or deletion as described elsewhere herein.

10   Covalent modification may accomplish essentially the same objective as a site-directed mutant at the same location using a naturally occurring residue.

Tryptophan is a relatively rare amino acid in hOAT. Accordingly, this residue is an attractive site for post-translational convalent modification because substitution at other sites is expected to be less than may be the case

15   with more common residues. Reaction of trp with an oxidant such as a halogen donor, e.g. bromine, will yield the side chain structure
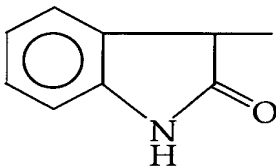
This reaction should be conducted in aqueous solvent and at low halogen

20   concentrations.

Other covalent modifications of hOATs will be apparent to the artisan. Sensitive side chains are protected by masking them with antibodies directed against an epitope that includes the residue to be protected. Reagents for accomplishing such modifications are well-known and have been widely used

25   in the diagnostic and preparative fields. See T. Creighton, *Proteins: Structure and Molecular Properties*, 1983.

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hOATs are cross-linked to a water insoluble matrix or incorporated into a lipid vehicle such as a liposome, usually a ULV. Cross-linking is accomplished by reacting the hOAT and matrix with a bifunctional agent. Examples of suitable bifunctional agents include 1,1-bis(diazoacetyl)-2-

5 phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dethiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield

10 photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, hOAT is immobilized on reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440.

15   hOATs also are covalently modified by linking the hOAT to various nonproteinaceous polymers, e.g., polyethylene glycol (PEG), polypropylene glycol or polyoxyalkylenes, in the manner set forth for example in U.S. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337. PEG is a non-immunogenic, linear, uncharged polymer with three water molecules per

20 ethylene oxide unit. See Maxfield, et al., "Polymer" 16:505-509 (1975); Bailey, F. E. et al., in "Nonionic Surfactants", Schick, M. J., ed, pp. 794-821 (1967). Several therapeutic enzymes have been conjugated to PEG to increase their *in vivo* half-life (Abuchowski, A., et al., "J. Biol. Chem." 252:3582-3586 (1977); Abuchowski, A. et al., "Cancer Biochem. Biophys." 7:175-186 (1984). An IL-2 (interleukin-2)-

25 PEG conjugate has been reported to increase circulatory life and potency (Katre, N.V. et al., "Proc. Natl. Acad. Sci." 84:1487-1491 (1987); Goodson, R. et al., "Bio/Technology" 8:343-346 (1990)). See also Abuchowski, A. et al., "J. Biol. Chem." 252:3578-3581 (1977). Any of the methods for PEG conjugation used in these citations is acceptable for use with the hOATs of this invention.

Uses for the hOATs of this Invention

The hOATs of this invention are useful in therapeutic, diagnostic and
preparatory methods. Their use will depend upon the properties that they
possess, as will be apparent to the ordinary artisan. For the most part, all of the
hOATs will retain hOAT immune epitopes, so they are useful in place of hOAT
in hOAT immunoassays whether or not they possess any anion transport
activity.

The hOATs of this invention are useful in identifying substances that
bind to hOAT (hOAT binding partners, or "TBP"). Of particular interest are
substances that are capable of binding to hOAT to substantially inhibit the
anion transport activity of hOAT, or, preferably, to introduce favorable
selectivity into the transport activity such that nephrotoxic drugs such as
amphotericin or cidofovir are not taken up as avidly as in the absence of the
substance. TBP's ability to bind to hOAT also is useful in methods for
recovering hOAT from contaminated mixtures such as cell culture supernatants
of recombinant hOAT-expressing cells (including the hOAT amino acid
sequence hOATs herein). Typically, hOATs can be used in place of hOAT
standards in immunoassays for hOAT if they possess at least one hOAT epitope
recognized by the antibody used in the hOAT immunoassay in question, while
the TBPs are used in place of antibodies for hOAT. The hOATs also are useful,
as appropriate, in functional assays for certain individual properties of hOAT.

Peptide TBPs are obtained by the use of *in vitro* directed evolutionary
methods such as those employing filamentous phage to present candidate
sequences (otherwise known as phage display) and similar methods known *per
se* which rely on the systematic generation and screening of peptides for
activity. These typically are rather small molecules, containing on the order of
about 5 to 20 residues.

Antibody TBPs are immunoglobulins, ordinarily monoclonal antibodies, which (in preferred embodiments) are capable of specifically inhibiting the transport function of hOAT. Antibodies are raised in conventional fashion by immunizing an animal with an immunogenic hOAT conjugate, e.g. hOAT

5   crosslinked to keyhole limpet hemocyanin.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e. the individual antibodies comprising the population are essentially identical in specificity and affinity. Monoclonal antibodies include hybrid and recombinant

10   antibodies (e.g. "humanized" antibodies) regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, $F(ab')_2$, and Fv). Thus, "monoclonal" antibodies are produced by any particular method that will yield a substantially homogeneous population. For example, monoclonal antibodies may be made using the methods described by

15   Kohler & Milstein, "Nature" 256:495 (1975), Goding, Monoclonal Antibodies: Principles and Practice pp. 59-103 (1986), Kozbor, "J. Immunol." 133:3001 (1984), or Brodeur, et al., Monoclonal Antibody Production Techniques and Applications, pp. 51-63 (1987), or may be made by recombinant DNA methods. Cabilly, et al., U.S. Pat. No. 4,816,567.

20   In a preferred embodiment of the invention, the monoclonal antibody will have an affinity for reference sequence hOAT of at least about 10^9 moles/liter, as determined, for example, by the Scatchard analysis of Munson & Pollard, "Anal. Biochem." 107:220 (1980). Also, the monoclonal antibody typically will inhibit the transport activity of hOAT (using a standard organic

25   anion such as para-aminohippurate) by at least about 50%, preferably greater than 80%, and most preferably greater than 90%, as determined by conventional methods.

DNA encoding the monoclonal antibody is readily isolated and
sequenced using conventional procedures (e.g., by using oligonucleotide probes
that are capable of binding specifically to genes encoding the heavy and light
chains of murine antibodies). Hybridoma cells serve as a preferred source of

5   such DNA. Once isolated, the DNA may be placed into expression vectors,
which are then transfected into host cells such as simian COS cells, Chinese
Hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce
immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in
the recombinant host cells.

10   The DNA optionally may be modified in order to change the character of
the immunoglobulin produced by its expression. For example, humanized
forms of murine antibodies are produced by substituting a complementarity
determining region (CDR) of the murine antibody variable domain for the
corresponding region of a human antibody. In some embodiments, selected

15   framework region (FR) amino acid residues of the murine antibody also are
substituted for the corresponding amino acid residues in the human antibody.
Humanized forms of murine antibodies also may be produced by substituting
the coding sequence for human heavy and light constant chain domains in place
of the homologous murine sequences. Morrison, et al., "PNAS" 81:6851 (1984).

20   TBP also includes nucleic acid sequences that bind hOAT. Evolutionary
selection methods for oligonucleotides that bind to target proteins are well
known (WO 92/14843; Ellington et al., "Nature" 355:850 (1992); Bock et al.,
"Nature" 355:564 (1992); Ellington et al., "Nature" 346:818 (1990); Tuerk et al.,
"Science" 249:505 (1990). These oligonucleotides, commonly known as

25   aptamers, generally contain the usual A, T, G, C or U bases or derivatives
thereof, and comprise sequences that bind to a predetermined site on a target
protein. A selection method for TBPs that inhibit the transport function of
hOAT comprises (a) preparing a pool of candidates (oligonucleotides, peptides,
extracts, proteins, etc.), (b) contacting the candidates with hOAT having anion

transport activity (typically reference sequence hOAT) (c) isolating from the hOAT those candidates that are able to bind to hOAT, (d) contacting the candidates from step c) with an hOAT in which the hOAT transport function has been mutated substantially out of the hOAT, and (e) recovering those

5   candidates that do not bind to the mutated form.

For diagnostic applications, the hOATs of this invention optionally are labeled with a detectable moiety, either *in vivo* or *in vitro*. The detectable moiety can be any substituent which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a

10   radioisotope, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, or ^{125}I, a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; a radioactive isotopic label, such as, ^{125}I, ^{32}P, ^{14}C, or ^{3}H, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase.

Any method known in the art *per se* can be used to conjugate the hOAT

15   to the detectable moiety. See the methods described *supra* and Hunter, et al., "Nature" 144:945 (1962); David, et al., "Biochemistry" 13:1014 (1974); Pain, et al., "J. Immunol. Meth." 40:219 (1981); and Nygren, J. "Histochem. and Cytochem." 30:407 (1982). Oligonucleotide TBPs are labeled in the conventional fashion heretofore employed in the diagnostic probe art.

20   The TBPs or hOATs of the present invention optionally are employed in known immunoassay techniques, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, Monoclonal Antibodies: A Manual of Techniques, pp. 147-158 (1987).

Competitive binding assays rely on the ability of a labeled standard

25   (which may be hOAT, or an immunologically reactive portion thereof such as a labeled hOAT of this invention) to compete with the test sample hOAT for binding with a limited amount of TBP. The amount of hOAT in the test sample is inversely proportional to the amount of standard that becomes bound to the

TBP. To facilitate determining the amount of standard that becomes bound, the TBP generally is insolubilized before or after the competition, so that the standard and analyte that are bound to the TBP conveniently are separated from the standard and analyte which remain unbound.

5   Sandwich assays involve the use of two TBPs, each capable of binding to a different target portion, or epitope, of hOAT. In a sandwich assay, the test sample analyte is bound by a first TBP which is immobilized on a solid support, and thereafter a second TBP binds to the analyte, thus forming an insoluble three part complex. David & Greene, U.S. Pat No. 4,376,110. The second

10   antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-TBP antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

15   The TBPs of this invention also are useful for *in vivo* imaging, wherein a TBP labeled with a detectable moiety is administered to a host, preferably into the bloodstream, and the presence and location of the labeled TBP in the host is assayed.

hOAT nucleic acids or TBPs also can be used in a method for identifying

20   isoforms or alleles of hOAT nucleic acids or polypeptides that place a patient at particular risk for nephrotoxicity. Such variants, alleles or isoforms will exhibit different selectivity towards uptake of certain anions. If these anions are nephrotoxic drugs or drug metabolites, then a patient may be at particular risk of injury. The patient can be tested (e.g., by amplifying genomic hOAT-

25   encoding DNA from a blood sample) to determine if the at-risk variant, isoform or allele is present. If so, then the dosage of the drug can be reduced or an alternative drug used. This method comprises identifying one or more naturally-occurring sequence variations in the human population and determining the selectivity of such variations for transport of a selected

nephrotoxic substance. Then a patient is tested for the variation associated with relatively selective transport of the nephrotoxic substance.

Similarly, allelic variation in the transcriptional control regions of the hOAT gene can be analyzed for correlation with susceptibility to uptake and transport of selected nephrotoxic drugs; here the correlation will be with the amount of expression of hOAT as opposed to the selectivity of the hOAT protein. This method comprises identifying one or more naturally-occurring sequence variations in the expression control domain of hOAT in the human population and determining the expression levels of hOAT in cells containing such variant. Patients having relatively high levels of expression of hOAT may not be considered suitable for treatment with nephrotoxic drugs, or the dosage of such drugs may be reduced.

Since at least a domain of hOAT is expressed in the brain as well as the kidney (cf brain EST R25797 and Example 3 below), it is also within the scope of this invention to probe a brain library to obtain the full length gene corresponding to R25797. The sequence of this gene may or may not diverge substantially from the reference sequence hOAT. This notwithstanding, the brain-expressed gene is also a suitable subject for determination of sequence variants such as alleles and isoforms. These may be involved in important neural functions and are suitable subjects for screening to identify them.

hOAT also is useful in screening methods for compounds that can act to suppress or enhance anion uptake and transport by hOAT. Suppression of hOAT activity (antagonism) is useful in reducing the nephrotoxicity of drugs, i.e., to serve as nephroprotective agents, while enhancement of hOAT activity (agonism) is useful in the treatment of kidney dysfunction. The screening methods comprise providing a candidate hOAT agonist or antagonist, contacting the candidate with hOAT nucleic acid or polypeptide, determining the effect of the candidate on the transcription of the hOAT nucleic acid (or the expression or biological activity of the hOAT polypeptide), identifying a hOAT

agonist or antagonist, and optionally preparing additional quantities of the agonist or antagonist that is so identified. Biological activity of hOAT is assayed using the stable cell line of Example 1 as shown in Example 2.

hOAT is useful in screening assays for modified forms of heretofore

5 nephrotoxic drugs, or as part of a toxicology screening program to determine the potential nephrotoxicity of new therapeutic compounds. Accumulation of the candidate in hOAT transformants as opposed to control cells is an indicia of potential nephrotoxicity. Determining CC_{50}'s using the hOAT transformants would produce a direct measure of potential nephrotoxicity. A candidate form

10 of a suspected compound or a known nephrotoxic drug, e.g., a prodrug, is contacted with the hOAT and its effect on the transport by hOAT of a benchmark anion is determined. Transport of the test compound itself (usually in an isotopically labeled form) also can be determined. Alternatively, transport of the compound in the presence of probenecid can be assayed. If the

15 compound is no longer transported, or the benchmark anion transport is not affected by the compound, or probenecid has no influence on whether or not the compound is transported, then the candidate is suitable for further study in animals as a potential non-nephrotoxic drug, for example, in the case of a prodrug as a non-nephrotoxic form of the parental drug. Prodrugs of

20 nucleotide phosphonate analogues such as cidofovir, PMEA or PMPA suitable for testing include mono and di-esters and amidates of the phosphonyl group.

Example 1. - *Generation of a cell line stably transformed with hOAT1 gene*

A cell line stably expressing hOAT1 was prepared and characterized to ultimately prove that the isolated cDNA encodes functional hOAT1 protein.

The expression construct containing hOAT1 gene was prepared as follows. hOAT1 coding sequence was PCR amplified from plasmid isolated from human kidney cDNA library. PCR reaction was performed under standard conditions using Expand High Fidelity PCR system (Boehringer Mannheim). Oligonucleotides 5'-ACCGTCTAGAATTCTTTTTATTTTTAATTTTCTTTCAAATACGTCCACCAT GGCCTTTAATGACCTCCTGCAGCAGG-3' (SEQ. ID NO. 3) and 5'-TACTCACGTGGATCCTGATCAGACGTCTGTAGGACCTTCCCTCCCTTTAG G-3' (SEQ. ID NO. 4) were used as a sense and antisense primer to introduce EcoRI and BamHI restriction site, respectively. In addition, the sense primer contained truncated 5'-untranslated sequence of alfalfa mosaic virus (underlined) and favorable Kozak consensus sequence (CCACCATGG) (SEQ. ID NO. 5) to maximize initiation of translation. The PCR product was digested with EcoRI/BamHI and cloned into pIRESneo expression vector (CLONTECH, Palo Alto, CA) using the corresponding restriction sites to yield pIRES-hOAT plasmid. After cloning, the correct nucleotide sequence of the whole fragment generated by PCR was verified.

pIRES-hOAT plasmid was transfected into CHO-K1 cells (ATCC CCL61) growing in F-12 nutrient mixture supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. 24 hours before transfection, approximately 6x10^6 CHO-K1 cells were seeded into 100-mm Petri dish. At the time of transfection, media was aspirated and 6 ml of fresh media containing 12 μg of pIRES-hOAT and 60 μg of Cytofectin GSV cationic lipid (Glen Research, Sterling, VA) was added to the cells. Following overnight incubation, selection of stable transformants started in phenol red-free F-12 nutrient mixture containing fetal bovine serum, penicillin and streptomycin as above, plus 1

mg/ml G418 (CLONTECH). Viable colonies were isolated after 2-week

selection and tested for uptake of p-aminohippuric acid (PAH, a prototype

substrate of organic anion transport systems) in the presence and absence of

probenecid (inhibitor of organic anion transport). For the PAH uptake assay,

5   the cells were seeded into 12-well plates at the density of $2x10^5$ cell/well. After

48 hours, growth media was aspirated and the cells were washed twice with

phosphate buffered saline (PBS). The uptake assay was performed in transport

buffer (135 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.8 mM $MgSO_4$,

28 mM glucose, and 13 mM HEPES pH 7.2) containing 5 µM [^{3}H]PAH (New

10   England Nuclear, spec. activity 1.2 Ci/mmol) ± 1 mM probenecid. After 90 min

incubation at 37°C in the transport buffer, the cells were washed 3-times with

ice-cold PBS (2 ml/well) and lysed directly on the plate in the presence of 0.3%

Triton X-100 (0.5 ml/well) for 20 min at room temperature. Plates were washed

with additional 0.5 ml/well of Triton X-100, the lysate and wash were

15   combined, scintillation fluid was added, and the radioactivity counted. As a

result of this assay, a transformed clone with 30-fold increase in PAH uptake

compared to parental CHO-K1 cells was identified and designated CHO-hOAT.

Example 2 - *Functional characterization of hOAT1*

20   Kinetics of hOAT1-mediated PAH uptake was characterized using CHO-

hOAT cells. The uptake assay was performed as described above with various

concentrations of [^{3}H]PAH ranging from 5 to 160 µM. To assess net hOAT1-

specific uptake, background uptake measured in parental CHO-K1 cells at each

substrate concentration was subtracted from that determined in CHO-hOAT

25   cells. Kinetic constants were calculated using Enzyme kinetics software

(ChemSW, Fairfield, CA). As expected, PAH uptake in CHO-hOAT cells was

saturable with K_m = 13 µM and Vmax = 42 pmol/10^6 cells. As oppose to the

background uptake of PAH in CHO-K1 cells, uptake in CHO-hOAT cells was

strongly sensitive to probenecid with IC_{50} = 6.2 μM when measured at PAH

concentration equal to its K_m. In addition, PAH accumulation in CHO-hOAT

was stimulated approximately 2.5-fold by preloading the cells with 10 mM

glutarate indicating that the hOAT protein functions as an organic

5   anion/dicarboxylate exchanger.

Example 3 - *Tissue distribution of hOAT1*

A human multiple tissue Northern blot (CLONTECH) was used for

localization of hOAT1 expression in human tissues. A hOAT1-specific

10   [^{32}P]dATP labeled probe was generated by random priming the BsrGI/Bsu36I

DNA fragment corresponding to nucleotides 420-854 of the hOAT1 coding

sequence. The membrane was hybridized for 1 hour at 68°C in ExpressHyb

hybridization buffer (CLONTECH) and then washed twice in 2X SSC with

0.05% SDS for 30 minutes at room temperature followed by a single wash in

15   0.1X SSC with 0.1% SDS at 50°C. A strong signal was detected in kidney

corresponding to a 2.5 kb hOAT1 transcript. No positive signal was found in

the other tested tissues (brain, heart, skeletal muscle, colon, thymus, spleen,

small intestine, placenta, lung, or peripheral blood leukocytes).

In addition, hOAT1 expression in various human tissues was examined

20   by more sensitive RT-PCR amplification. For this purpose, the Multiple Choice

cDNA kits I and II containing tissue specific cDNAs (Origene Technologies,

Rockville, MD) and two sets of hOAT1-specific primers were used for PCR

detection of hOAT1 expression. The oligonucleotides,

5'-CCCGCTGGCACTCCTCCTCCGGGAG-3' (SEQ. ID NO. 6)

25   (sense), and

5'-GTAGAGCTCGGCAGTCATGCTCACCA-3' (SEQ. ID NO. 7)

(antisense), was used to amplify a 606-bp fragment from the hOAT1 coding

region (nucleotides 815-1420). In the independent set of PCR reactions, a 295-bp

hOAT1 fragment (comprised of the last 175 coding nucleotides and 120

nucleotides of the 3′-UTR) was amplified using the oligonucleotides,

5′-CCAGCGCTGTCACTGTCCTCCTGC-3′ (SEQ. ID NO. 8)

(sense), and

5   5′-AACCCCCACACTTGGGTCACCATTTCCTC-3′ (SEQ. ID NO.

9) (antisense). PCR reactions were carried out using the Expand High Fidelity

PCR System (Boehringer Mannheim) in a total volume of 25 μl containing 1 μg

of tissue-specific cDNA. Thirty-five amplification cycles (95°C for 40 s, 58°C for

1 min, and 72°C for 45 s) were performed. Positive tissues were identified after

10   separation of PCR reactions on a 1% agarose gel. As expected, a strong positive

signal was consistently detected in kidney. In contrast with the Northern

analysis, brain and skeletal muscle was also positive for hOAT1 expression

although to lesser extent compared to kidney.

Presence of hOAT protein in human kidney tissue was verified by an

15   immunoblot analysis with rabbit anti-hOAT1 polyclonal antibody prepared at

AnaSpec (San Jose, CA) using standard immunological techniques. Briefly, an

immunogenic peptide, NH$_2$-TVQDLESRKGKQTR-COOH (SEQ. ID NO. 10),

corresponding to hOAT1 amino acids 515-528 was conjugated to keyhole limpet

hemocyanine through a cysteine residue added to the peptide's C-terminus.

20   Animal serum was collected after 4 immunizations in the presence of complete

Freund's adjuvant and affinity-purified against the immunogenic peptide

immobilized on a Sepharose resin. For immunoblot analysis, human kidney

cortex was extractracted with a buffer containing 20 mM Tri-HCl, pH 7.5, 150

mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS. After homogenization

25   in the presence of complete proteinase inhibitor cocktail (Boehringer

Mannheim) the extract was clarified by high-speed centrifugation, separated by

electrophoresis on an 8% SDS-polyacrylamide gel and electroblotted onto a

nitrocellulose membrane (Millipore, Bedford, MA). The membrane was

blocked in PBS/5% dry milk (PBS-M) for 1 hour, washed 3 times in PBS/0.05%

Tween 20 (PBS-T), and incubated overnight in PBS-M with the anti-hOAT

antibody. Following wash in PBS-T, the membrane was incubated in PBS-M

with goat anti-rabbit antibody conjugated to horseradish peroxidase (Zymed,

5   South San Francisco, CA). After an additional wash and incubation with a

chemiluminescent substrate (Amersham, Arligton Heights, IL), the immunoblot

was exposed to X-ray film. The antibody recognized a heterogeneous product

with an apparent molecular weight of 80 to 90 kDa. This was significantly

larger than the predicted molecular weight from the hOAT1 amino acid

10   sequence (60.3 kDa). However, when the cortex extract was treated with

peptide:N-glycosidase F, which specifically cleaves N-linked oligosaccharide

chains, a homogeneous product of 60 kDa was detected on the immunoblot.

Example 4 – *Determination of Nephrotoxic Potential by Cell Culture Assay Using*

15   *hOAT Transformants*

Adefovir (ADV) is an anti-HIV nucleotide analog with unique resistance

profile currently undergoing Phase III clinical evaluation. The most important

clinical toxicity of ADV is nephrotoxicity associated with changes in laboratory

markers of renal functions. ADV is a substrate for human renal organic anion

20   transporter 1 (hOAT) located in the basolateral membrane of the proximal

convoluted tubules. In this example, the role of hOAT in the mechanism of

ADV nephrotoxicity was investigated.

Chinese hamster ovary cells (CHO), which exhibit low sensitivity to

ADV cytotoxicity due to its limited uptake, were stably transformed with hOAT

25   cDNA to generate CHO-hOAT cells as described above. Uptake and

cytotoxicity of ADV in the two cell lines was compared.

CHO-hOAT accumulated ADV to levels >300-fold higher compared to

CHO. Uptake of ADV by CHO-hOAT was saturable (K_m = 23 uM, V_{max} = 390

pmol/10^6 cells) and sensitive to the hOAT inhibitor probenecid (PBC; IC_{50} = 6.5

uM). Importantly, ADV was ~500-fold more cytotoxic in CHO-hOAT compared to CHO cells. However, in the presence of 1 mM PBC, CHO-hOAT were only 3-fold more susceptible to ADV than were CHO. Another antiviral nucleotide, cidofovir (CDV), but not its cyclic prodrug (cCDV), also efficiently accumulated

5   in CHO-hOAT (K_m = 70 uM, V_{max} = 1,110 pmol/10^6 cells). Accordingly, CDV was >400-fold more cytotoxic in CHO-hOAT compared to CHO. In contrast, cytotoxicity of cCDV was increased to much lesser extent in CHO-hOAT cells corresponding with the lack of cCDV nephrotoxicity. Similar to ADV, CDV cytotoxicity was also significantly reduced by PBC.

10   Expression of hOAT enhances cytotoxicity of ADV. Since high-level expression of hOAT is specific to renal tubules, hOAT plays a crucial role in the mechanism of ADV nephrotoxicity. Thus, hOAT inhibitors (e.g. PBC) may be useful to overcome ADV nephrotoxicity. Observations with CDV and cCDV correlate with their nephrotoxic potential and provide additional support for

15   the involvement of hOAT in this process.